b.) Remarks

Claims 82-87 are amended more specifically to recite the subject matter of the present invention.

The Examiner's efforts to vacate the October 4, 2007 Official Action are very gratefully acknowledged. Meanwhile, Applicants have completed their experiments and obtained an executed Declaration under Rule 132 of Dr. Hiromasa Miyaji addressing the Examiner's prior concerns. That executed Declaration is attached hereto.

In the December 28, 2006 Advisory Action the Examiner stated

> "In addition, Applicants failed to overcome the claim 81 rejection under 35 USC 102(b) as being anticipated by Thompson, because even though Thompson uses mouse embryonic fibroblasts as feeder cells the claimed invention does not preclude the use of any stroma cell which is recognized by a monoclonal antibody produced by hybridoma FERM BP-7573."

As the Examiner is aware, Applicants earlier distinguished the prior art by pointing out the present invention utilizes a stroma cell recognized by the hybridoma produced by FERM BP-7573. The Examiner maintained such was inherent to the cited art and so Applicants sought to provide the attached determinative evidence.

As discussed in Dr. Miyaji's Declaration, FERM BP-7573 produces monoclonal antibody KM1310. In order to squarely address the Examiner's concern, Applicants' experiments provide conclusive evidence that Thompson's mouse embryonic

fibroblast cells do not express KM1310 antigen and are not recognized by a monoclonal antibody produced by FERM BP-7573. See page 3 of the Declaration, last 10 lines.

That point is now addressed.

Additionally, in the Advisory Action[1], the Examiner stated

> "claims 1 and 80 as amended do not override the rejection of claims 1, 4, 18-24, 26, 27, 72, 74-75, 80, 82-87 under 35 USC 112, 1st paragraph, written description. The present claims as have been amended still stand open-ended and allows for additional steps. That is the claims encompass a method that has culturing an embryonic stem cell for 1 to 14 days, and then culturing continuously." It is noted that the clause "wherein" does not preclude the use of any stroma cell which is recognized by monoclonal antibody produced by hybridoma FERM BP-7573.

Applicants' August 17, 2007 Preliminary Amendment was intended to address these concerns. However, in the vacated October 4, 2007 Office Action, the Examiner rejected claims 1, 15, 18-21, 23, 24, 72, 74, 75 and 80-87 under 35 USC §112 as failing to comply with the written description requirement and stated there is no literal support for culturing an embryonic stem cell *in vitro* (i) in the absence of retinoic acid and (ii) in the presence of stroma cell recognized by a monoclonal antibody produced by FERM BP-7573 without forming embryoid body for 1 to 14 days, and then continuously culturing the cell *in vitro* (iii) in the absence of retinoic acid and (iv) in the presence of both BMP-4 and a stroma cell recognized by a monoclonal antibody produced by FERM BP-7573 without forming embryoid body.

In response, Applicants wish to point out that page 30, lines 27-30 explicitly teaches that

[1] Also in the vacated October 4, 2007 Office Action.

> "Examples of the nervous system cell include a neural stem cell, a nerve cell, a cell of neural tube, a cell of neural crest and the like."

These cells are explained with greater particularity from page 33, line 31 to page 34, line 27

> **Cells of neural tube and neural crest** In the initial stage development in chordates, a primitive streak appears in the primitive ectoderm layer and neural induction starts. The neural induction means a stage in which an ectoderm positioned on the dorsal side of an early embryo receives a signal from an organizer region positioned in its adjacent or inner part and thereby differentiates into a neuroectoderm. The neuroectoderm formed by this neural induction becomes a neural plate independently from a non-neuroectoderm, namely an epidermal ectoderm, and then forms a neural tube by invaginating into the ventral side. The ectoderm portion positioned between the neural plate and epidermal ectoderm forms a neural crest during the invagination. All cell groups of the central nervous system are generated from one layer of the neuroepithelial tissues which constitute the neural tube. That is, the front part of the neural tube expands and forms a brain vesicle which becomes primordium of the brain, and the rear part differentiates into the spinal cord as the tube. The neural crest does not directly take part in the differentiation of central nerve itself, but the cells constituting neural crest migrate and differentiate into various tissues such as cerebral or spinal ganglion, sympathetic nerve and its ganglion, adrenal medulla and melanocyte.

- Thus, the specification explains that neural induction commences from a primitive streak in the primitive ectoderm layer. Neuroectoderms become a neural plate independent from an epidermal ectoderm and then form a neural tube by invagination into the ventral side. During invagination the ectoderm portion between the neural plate and the epidermal ectoderm forms a neural crest.

The specification from page 34, line 28 to page 35, line 13, sets forth the process for producing neural stem and neural crest cells.

> The cell of neural tube induced from an embryonic stem cell by the method of the present invention includes a cell characterized as a cell of neural tube before the step in which the dorso-ventral axis is determined, which is capable of differentiating into a cell positioned at the ventral side by reacting with sonic hedgehog (hereinafter referred to as "shh") as a ventral factor of neural tube and of differentiating into a cell positioned at the dorsal side by reacting with bone morphogenetic protein 4 (hereinafter referred to as "BMP4") as a dorsal factor of neural tube. Also, a cell of the neural tube ventral side, expressing a marker HNF-3β (hepatocyte nuclear factor-3 β, hereinafter referred to as "HNF-3β") positioned on the basal plate of the most ventral side of neural tube, a cell of the neural tube ventral side, expressing a marker Nkx2.2 existing secondary to the HNF-β from the ventral side of neural tube, and a cell of the neural tube dorsal side, expressing Pax-7, all of which are differentiated from the above cell, are also included as neural tube cells induced from an embryonic stem cell by the method of the present invention.

- Thus, it is seen the specification teaches neural tube cells can be induced from (i) embryonic stem cells before the dorso-ventral axis is determined which differentiate into ventral side cells upon reaction with sonic hedgehog, (ii) embryonic stem cells before the dorso-ventral axis is determined which differentiate intodorsal side cells upon reaction with BMP4, (iii) ventral side neural tube cells expressing marker HNF-3β, ventral side neural tube cells expressing marker Nkx2.2, and (iv) dorsal side neural tube cells expressing Pax-7.

The foregoing was all put into place in Example 14 from page 114, line 30 to page 117, line 28, which teaches the following:

Differentiation induction of embryonic stem cell into various neural cells along the dorso-ventral axis

In order to examine effects of shh and BMP4 as factors which determine diversity of nerves along the dorso-ventral axis in the generation of central nervous system, these factors were allowed to act upon an ES cell which started its differentiation on a stroma cell and their influences were examined in the following manner.

According to the method described in Example 1, the ES cell EB5 was cultured for 10 days in the serum-free medium without BMP4 using the PA6 cell as a feeder cell. That is, the PA6 cell proliferated to almost confluent on a 3 cm tissue culture dish was used as a feeder cell, the ES cell was inoculated onto the feeder cell at a density of 200 cells/dish, the medium was exchanged using a fresh serum-free medium on the 4th, 6th and 8th days, and the cells were cultured at 37ºC for 10 days in a 5% CO_2 incubator.

Effects of shh were evaluated using a serum-free medium to which 300 nmol/l of shh (manufactured by R & D) had been added at the time of the medium exchange on the 4th, 6th and 8th days.

Effects of BMP4 were evaluated using a serum-free medium to which 0.5 nmol/l of BMP4 (manufactured by R & D) had been added at the time of the medium exchange on the 4th, 6th and 8th days.

Ten days after of coculturing, the cells cultured by respective culturing methods were fixed according to the method described in Example 1, and the colonies formed as a result of coculturing of the ES cell with PA6 cell were immunologically stained using the anti-NCAM antibody, an antibody against HNF-3β which is a marker of the basal plate existing on the most ventral side of the central nervous system primordium (neural tube) (purchased from Developmental Studies Hybridoma Bank), an antibody against Nkx2.2 as a marker existing secondary to the HNF-3β from the ventral side (purchased from Developmental Studies Hybridoma Bank), an

antibody against Pax-7 as a marker of the neural tube dorsal side (purchased from Developmental Studies Hybridoma Bank), an antibody against AP-2 as a marker of the neural crest cell (purchased from Developmental Studies Hybridoma Bank), an antibody against islet 1 as a marker of motor neuron (purchased from Developmental Studies Hybridoma Bank) and an antibody against VAchT which is a marker of cholinergic neuron (manufactured by Chemicon).

Regardless of the addition of shh or BMP4, most of the colonies formed as a result of coculturing of the ES cell EB5 with PA6 cell were stained with the anti-NCAM antibody similar to the results shown in Example 1, and 90% of the ES cell-derived colonies were positive in both cases.

The result is shown in Table 1, together with the ratio of ES cell-derived colonies stained with antibodies against other markers.

1 TABLE 1 Antibodies Control shh added BMP4 added Anti-NCAM antibody 90% 90% 90% Anti-HNF-3β antibody 70% 81% 9% Anti-Nkx2.2 antibody 44% 85% 19% Anti-Pax-7 antibody 30% 0% 72% Anti-AP-2 antibody 16% 0% 24% Anti-islet 1 antibody 82% 82% 36% Anti-VAchT antibody 36% 58% 42%

It was shown from the above results that nervous system cells expressing not only the NCAM as a neuron marker but also various types of neuron-specific markers are formed by the nerve cell induction of the ES cell by its coculturing with the PA6 cell. That is, when the ES cell was differentiation-induced by coculturing with the PA6 cell, it is differentiation-induced into a nervous system cell which is positioned on the basal plate of the most ventral side of the central nervous system primordium (neural tube) and expresses HNF-3β, a nervous system cell which is positioned secondary to the HNF-3β from the ventral side of the central nervous system primordium (neural tube) and expresses Nkx2.2, a nerve cell of the neural tube dorsal side expressing Pax-7, a neural crest cell expressing AP-2 and a motor neuron expressing islet 1. (Emphasis added.)

> Also, since shh and BMP4, whose relation to the determination of dorso-ventral axis during the embryo neurogenesis has been revealed, showed a differentiation potency similar to the in vivo differentiation potency of embryonic neural precursor cell, a cell of neural tube before the step of determining dorso-ventral axis is induced by coculturing ES cell with the PA6 cell. That is, in this neural tube cell, expression induction of the ventral side markers HNF-3.beta. and Nkx2.2 and expression inhibition of the dorsal side markers Pax-7 and AP-2 are observed by the action of shh as a neural tube dorso-ventral factor. On the other hand, when the BMP4 as a neural tube dorsal side factor is allowed to act, expression inhibition of the ventral side markers HNF-3.beta. and Nkx2.2 and expression induction of the dorsal side markers Pax-7 and AP-2 are observed.
>
> Also, a result similar to the above was obtained when a typical ES cell, 129 line mouse-derived CCE cell (M. R. Kuehn et al., Nature, 326, 295 (1987); Production of Mutation Mice Using ES Cell), was used

- Thus, it is seen that Example 14 explicitly shows producing neural tube and neural crest cells upon coculturing embryonic stem cells with stroma cells in the manner recited in the pending claims.

By the foregoing, it is apparent all the steps recited in claim 1 are all well-taught in the specification as filed. To the extent the Examiner objects to the claims because they could recite other steps, the specification clearly contemplates such other steps, for instance, including use of sonic hedgehog. Addition of sonic hedgehog is not recited in independent claim 1 and would therefore, to those of ordinary skill, be an "other step", as permitted by the open-ended claim language.

In view of the above amendments and remarks, Applicants submit that all of the Examiner's concerns are now overcome and the claims are now in allowable condition.

Accordingly, reconsideration and allowance of this application is earnestly solicited.

Claims 1, 15, 18-21, 23, 24, 72, 74, 75 and 8-87 remain presented for continued prosecution.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,

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